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Research Article

Rnd3 Regulation of the Actin Cytoskeleton Promotes Melanoma Migration and Invasive Outgrowth in Three Dimensions

R. Matthew Klein[1] and Andrew E. Aplin[1,2]

[1]Center for Cell Biology and Cancer Research, Albany Medical College, Albany, New York and [2]Department of Cancer Biology, Kimmel Cancer Center, Thomas Jefferson University, Philadelphia, Pennsylvania

Abstract

The depth of cell invasion into the dermis is a clinical determinant for poor prognosis in cutaneous melanoma. The signaling events that promote the switch from a noninvasive to invasive tumor phenotype remain obscure. Activating mutations in the serine/threonine kinase B-RAF are prevalent in melanoma. Mutant B-RAF is required for melanoma cell invasion. The expression of Rnd3, a Rho family GTPase, is regulated by mutant B-RAF, although its role in melanoma progression is unknown. In this study, we determined the functional contribution of Rnd3 to invasive melanoma. Endogenous Rnd3 was targeted for knockdown using a doxycycline-inducible short hairpin RNA system in invasive human melanoma cells. Depletion of Rnd3 promoted prominent actin stress fibers and enlarged focal adhesions. Mechanistically, stress fiber formation induced by Rnd3 knockdown required the specific involvement of RhoA and ROCK1/2 activity but not RhoB or RhoC. Rnd3 expression in human melanoma cell lines was strongly associated with elevated extracellular signal–regulated kinase phosphorylation and invasive behavior in a three-dimensional dermal-like environment. A functional role for Rnd3 was shown in the invasive outgrowth of melanoma tumor spheroids. Knockdown of Rnd3 reduced the invasive outgrowth of spheroids embedded in collagen gels. Additionally, Rnd3 depletion inhibited collective and border cell movement out from spheroids in a ROCK1/2-dependent manner. Collectively, these findings implicate Rnd3 as a major suppressor of RhoA-mediated actin cytoskeletal organization and in the acquisition of an invasive melanoma phenotype. [Cancer Res 2009;69(6):2224–33]

Introduction

A critical event during melanoma progression is the transition from radial growth phase to vertical growth phase. This transition is associated with movement out of the epidermis and invasion into the dermis (1). The molecular mechanisms underlying invasion in melanoma remain poorly understood. It is critical to understand these mechanisms because vertical growth phase cells acquire metastatic properties, and the depth of invasion is used as a clinical determinant of a poor prognosis.

Activating mutations in the serine/threonine kinase B-RAF occur at a frequency of 50% to 70% in melanoma and are detected throughout various disease stages (2, 3). Mutant B-RAF has been implicated with numerous cellular functions in melanoma including invasion through Matrigel (4, 5), a basement membrane-like extracellular matrix. Efficient activation of the mitogen-activated protein kinase/extracellular signal–regulated kinase (ERK) kinase (MEK)-ERK1/2 pathway downstream of mutant B-RAF mediates its effects on invasion. Noninvasive nevus cells and radial growth phase melanoma can harbor B-RAF mutations, but the MEK-ERK1/2 pathway is inefficiently activated due, in part, to negative feedback mechanisms (6, 7). When unabated, mutant B-RAF regulates melanoma cell invasion possibly by up-regulating expression of interstitial collagenase I/matrix metalloproteinase 1 (8, 9), although protease-independent invasion has also been reported in mutant B-RAF melanoma cells (10). Additional microenvironmental influences and alterations in the dynamics of the actin cytoskeleton and focal adhesions (extracellular matrix interaction sites) also contribute to motility and invasive processes (11–13); however, their role in mutant B-RAF regulated melanoma invasion remains poorly described.

Rho-family GTPases (RhoA, RhoB, RhoC, Rac, and Cdc42) are pivotal regulators of the actin cytoskeleton and cell migration (14, 15). Although rarely mutated, changes in their expression or functional activity have been associated with tumor progression (16, 17). Regulatory molecules that control Rho GTPase signaling are frequently responsible for the associated changes in actin cytoskeleton remodeling and tumor migration. Rnd3/RhoE/Rho8, a negative regulator of the Rho/ROCK pathway, has recently garnered attention as a prognostic indicator in cancer (18). Rnd3 is up-regulated in pancreatic cancer (19), melanoma (20, 21), and non–small cell lung cancer (22), whereas it is down-regulated in prostate cancer (23). Overexpression studies have implicated Rnd3 in disrupting actin stress fibers and focal adhesions in fibroblast and epithelial cells (24–27), yet conflicting roles remain for Rnd3 in transformation (28–30) and tumor cell migration and invasion (24, 31). The function of endogenous Rnd3 has been less well studied. Recent literature indicates that endogenous Rnd3 participates in ROCK-mediated apoptosis (32) and myoblast alignment (33).

We recently showed that mutant B-RAF expression in melanoma cells regulates actin cytoskeletal and focal adhesion organization (21). These effects were supported by mutant B-RAF control of Rnd3 expression, which acts as a regulator of cross talk between the B-RAF/MEK/ERK and Rho/ROCK/LIM kinase/cofilin pathways (21). In the current study, we show that endogenous Rnd3 influences actin architecture through specific attenuation of RhoA-stimulated actin stress fibers. Increased expression of Rnd3 correlated with the progression toward an invasive phenotype in melanoma cell lines. Notably, inducible depletion of Rnd3 by RNA interference decreased migration and invasive outgrowth on three-dimensional substrates. Collectively, these data highlight

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

Requests for reprints: Andrew E. Aplin, Department of Cancer Biology, Thomas Jefferson University, 233 S. 10th Street, Philadelphia, PA 19107. Phone: 215-503-7296; E-mail: Andrew.Aplin@KimmelCancerCenter.Org.

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the importance of Rnd3, a mutant B-RAF effector, to invasive melanoma behavior.

Materials and Methods

Cell culture. Human melanoma cells were provided by Dr. Meenhard Herlyn (Wistar Institute, Philadelphia, PA) and cultured as previously indicated (21). Neonatal foreskins were obtained according to Albany Medical College Institutional Review Board procedures. Normal human epidermal melanocytes were isolated and cultured as previously described (21).

Antibodies and reagents. The following antibodies were used: diphosphorylated (Thr18/Ser19) myosin light chain, kindly provided by Dr. Peter Vincent (Albany Medical College, Albany, NY; ref. 34); phospho-ERK1/2 (9106) from Cell Signaling Technology; ERK2, RhoA, RhoB, and RhoC from Santa Cruz Biotechnology; Rnd3 from Upstate Biotech, Inc.; and actin from Sigma-Aldrich. Alexa-Fluor–conjugated secondary antibodies were obtained from Molecular Probes Inc. TRITC-conjugated phalloidin and etoposide were purchased from Sigma-Aldrich.

Recombinant lentiviral infections. Inducible short hairpin RNA (shRNA) knockdowns were done using the BLOCK-iT lentiviral expression system (Invitrogen). Oligonucleotide sequences are listed in Supplementary Methods and were based on earlier studies using short interfering RNA (siRNA; ref. 21). All shRNA constructs and cell lines were generated as previously described (35). Constructs were verified by DNA sequencing. Inducible shRNA expression was achieved on addition of 100 ng/mL doxycycline (Fisher Scientific) to cell cultures.

siRNA knockdowns. The following siRNA duplexes, purchased from Dharmacon Research, Inc., were used: RhoA (AUGGAAAGCAGGUAGA-GUUUU), RhoB (ACACCGACGUCAUUCUCAUUU), and RhoC (GGAGAGAG-CUGGCCAAGAUUU). The nontargeting siControl$^{\#1}$ was also used (Dharmacon). Melanoma cells were transfected as previously indicated (21).

Western blotting. Standard methods were used for Western blotting using polyvinylidene difluoride membrane (21). Immunoreactive bands were developed using enhanced chemiluminescence kits (Pierce Chemical), detected with a Fluor-S MultiImager (Bio-Rad), and band intensity was quantified using Quantity-One image analysis (Bio-Rad).

Cell growth and cell cycle analysis. Experiments were done on cells in growth medium in the absence or presence of doxycycline. DNA synthesis was measured using Click-iT EdU Alexa Fluor 647 (Invitrogen). Briefly, knockdown cells labeled for 7 h with 10 μmol/L 5-ethynyl-2′-deoxyuridine (EdU) were resuspended, fixed, and processed according to the manufacturer's instruction. Propidium iodide staining was done using 10 μg/mL propidium iodide (Invitrogen). Flow cytometer was carried out using a BD FACSCanto. Data were analyzed with FlowJo cytometry software (Tree Star).

Immunofluorescence. Cells plated on glass coverslips were treated as previously indicated (21). An Olympus BX-61 microscope equipped with a charge-coupled device sensi-camera (Cooke) was used to photograph samples. Images were acquired using IPLab software (Scanalytics, Inc.). Quantification was done according to a previously outlined method (36) by categorizing at least 600 cells from multiple experiments depending on their cytoskeletal organization. Images were further processed using Adobe Photoshop Software (Adobe Systems).

Migration assays. Collective cell migration assays were done on spheroids placed on top of collagen I gels and submerged in complete growth medium. Cell movement was monitored for 5 h at 37°C. Images were acquired using an Olympus IX-70 inverted microscope equipped with a charge-coupled device sensi-camera (Cooke) and processed using Image-Pro Plus software (Media Cybernetics, Inc.). Collective cell migration was quantified using an integrated morphometric analysis to measure the change in spheroid surface area from identical spheroids. Migrating border cells were quantified by counting the number of individual cells separating away from the collective cell sheet during a 5-h time period.

Three-dimensional spheroid invasive outgrowth. Melanoma spheroids were prepared and implanted into collagen gel as described (37). Spheroids, formed by growing cells in nonadherent conditions for 72 h in the absence or presence of doxycycline, were embedded into bovine collagen I gels and placed into a dish precoated with a layer of acellular collagen. After the gel solidified, it was overlaid with normal growth medium, supplemented with doxycycline, if appropriate, and incubated for 4 d. To assess cell viability, spheroids were washed in PBS and incubated with calcein-AM and ethidium bromide according to the manufacturer's instruction (Molecular Probes, Inc.). Photographs were taken and processed for morphometric analysis as described above. A total of 8 to 20 spheroids from two to six experiments were analyzed per condition. Box-and-whisker plots indicate the 25th percentile (bottom line of the box), median (middle line), 75th percentile (top line of the box), 5th and 95th percentiles (whiskers), and minimum and maximum measurements (•) of the entire population.

Results

Rnd3 regulates the actin cytoskeletal organization of invasive melanoma. Rnd3 is a target of oncogenic B-RAF-MEK-ERK1/2 signaling in melanoma (21). To investigate the role of endogenous Rnd3 in melanoma, we constructed a doxycycline inducible shRNA system to knock down Rnd3 in WM793 and WM115 human invasive melanoma cell lines. WM793 and WM115 harbor the constitutive active B-RAF mutations V600E and V600D, respectively (38). To reduce concerns about "off-target" effects, experiments were done using two distinct Rnd3 shRNA (Rnd3$^{\#1}$ or Rnd3$^{\#2}$) sequences and a control nontargeting shRNA. Western blot analysis of multiple experiments indicated that treatment with doxycycline for 72 hours did not alter Rnd3 levels in control shRNA–expressing WM793 cells, whereas Rnd3$^{\#1}$ and Rnd3$^{\#2}$ shRNA lowered endogenous protein levels by 53% and 77%, respectively (Fig. 1*A* and *B*).

To investigate the consequence of reduced endogenous Rnd3 expression in invasive melanoma cells, we initially evaluated actin cytoskeletal organization. Microscopic evaluation of F-actin staining revealed that ~85% of control shRNA WM793 cells displayed either a disorganized or a cortical F-actin phenotype (Fig. 1*C* and *D*; Supplementary Fig. S1*A*). In contrast, endogenous Rnd3 depletion produced a switch to an actin phenotype composed predominately of stress fibers traversing the cell (Fig. 1*C* and *D*). Similar results were obtained following inducible knockdown of Rnd3 in the WM115 melanoma cell line (Supplementary Fig. S1*B* and *C*). Actin stress fiber formation following Rnd3 depletion required serum or growth factors because enhanced stress fiber formation was not observed in serum-free conditions (Supplementary Fig. S1*D*).

To further characterize the role of endogenous Rnd3, we evaluated whether alterations in focal adhesion localization accompanied Rnd3-dependent changes in actin organization. Inducible knockdown WM793 cells were processed for immunofluorescence analysis of vinculin, a marker of focal adhesions (39). We did not observe discernable differences in overall number of focal adhesions following Rnd3 knockdown; however, the size of focal adhesions was markedly enlarged (Supplementary Fig. S2*A*). Additionally, no alterations in cell surface integrin expression were detected (Supplementary Fig. S3). Collectively, these data suggest that endogenous Rnd3 may function to suppress serum- or growth factor–induced actin stress fiber and focal adhesion formation in invasive melanoma.

RhoA is required for Rnd3 knockdown–induced alterations in actin stress fibers. The appearance of actin stress fibers is a hallmark feature attributed to the activation of small GTPase Rho-family members (i.e., RhoA, RhoB, and RhoC; refs. 14, 26). To

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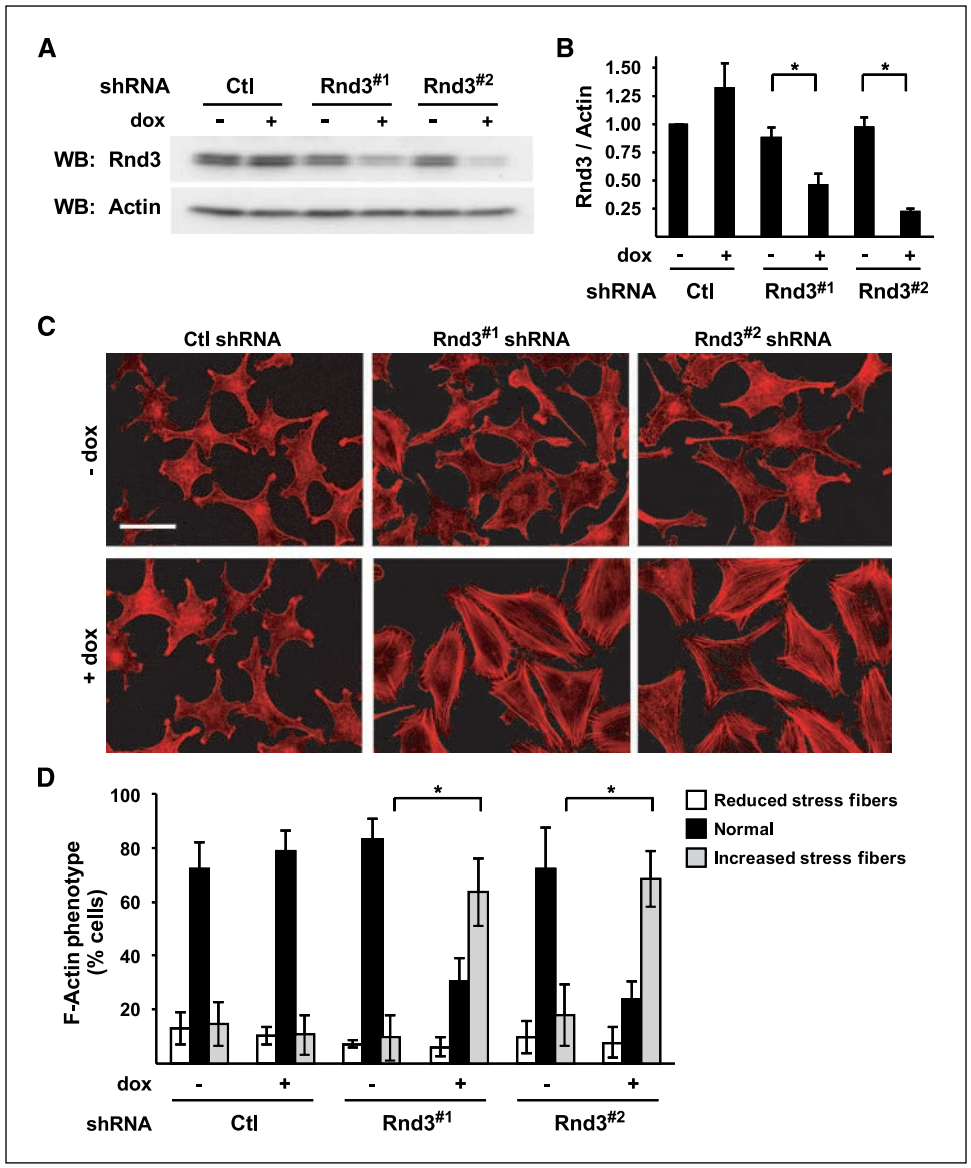

Figure 1. Depletion of endogenous Rnd3 induces melanoma actin cytoskeletal reorganization. Doxycycline-inducible melanoma cells expressing control (*Ctl*), Rnd3$^{\#1}$, or Rnd3$^{\#2}$ shRNA. *A,* cell lysates analyzed by Western blotting for Rnd3 and β-actin protein levels. *B,* quantitation of endogenous Rnd3 knockdown. *Columns,* mean Rnd3/actin ratios from three experiments with the control shRNA condition set to one; *bars,* SD. *, $P < 0.05$, Rnd3 knockdowns versus control knockdowns (two-tailed unpaired *t* test). *C* and *D*, F-actin organization in inducible shRNA melanoma cells. *C,* cells cultured +/− doxycycline (*dox*) for 72 h were fixed and stained with TRITC-phalloidin. *Bar,* 50 μm. *D,* quantitation of the F-actin phenotype shown in *C*. The graph displays the proportion of cells with visibly altered actin stress fiber organization; *columns,* mean of >600 cells from multiple independent experiments; *bars,* SD. *, $P < 0.05$.

establish which Rho isoform is required for stress fibers associated with Rnd3 depletion, we performed co-knockdown experiments. RhoA, RhoB, and RhoC isoforms are all expressed in WM793 cells and their protein expression did not change following Rnd3 depletion (Fig. 2*A*). Knockdown of individual Rho members by siRNA in the absence or presence of Rnd3 was confirmed by Western blot (Fig. 2*A* and data not shown). In cells expressing Rnd3, RhoA knockdown and, to a lesser extent, RhoC knockdown further reduced the already low levels of actin stress fibers (Fig. 2*B, left*). No apparent differences in actin organization were seen in RhoB knockdown cells. In Rnd3-depleted cells, enhanced actin stress fiber formation was antagonized by RhoA knockdown [Fig. 2*B* (*right*) and *C*]. Similar experiments using siRNAs targeting RhoB or RhoC did not produce any reversion of the Rnd3 knockdown phenotype. Rnd3 and RhoA have been implicated in regulating the activity of ROCK1. In non-muscle cell types, ROCK1/2 phosphorylates myosin light chain to promote the generation of cellular tension and actin stress fibers. Rnd3 depletion increased myosin light chain phosphorylation on Thr18/Ser19, which localized to actin stress fibers (Fig. 2*D*). Inhibition of ROCK1/2 using Y27632 reduced the Rnd3 knockdown increase in actin stress fibers and associated myosin light chain phosphorylation (Fig. 2*D*). Together these data suggest that Rnd3 expression in invasive melanoma suppresses the RhoA-ROCK1/2 pathway to promote actin cytoskeletal reorganization.

Rnd3 knockdown does not alter melanoma cell cycle. In addition to F-actin organization, Rnd3 has been implicated in cell proliferation (40) and survival (32). Therefore, we determined if Rnd3 expression regulates cell cycle progression in WM793 cells. Initially, DNA synthesis was monitored by flow cytometric evaluation of EdU incorporation (41). EdU incorporation was unaffected by Rnd3 knockdown (Fig. 3*A*). The overall cell cycle profile of inducible knockdown cells was then measured by propidium iodide staining under standard growth conditions. The DNA profile of Rnd3 knockdown cells was found to be similar to control cells (Fig. 3*B*). Analysis of signaling pathways linked to melanoma cell proliferation showed that ERK1/2 and AKT phosphorylation were similar despite Rnd3 knockdown

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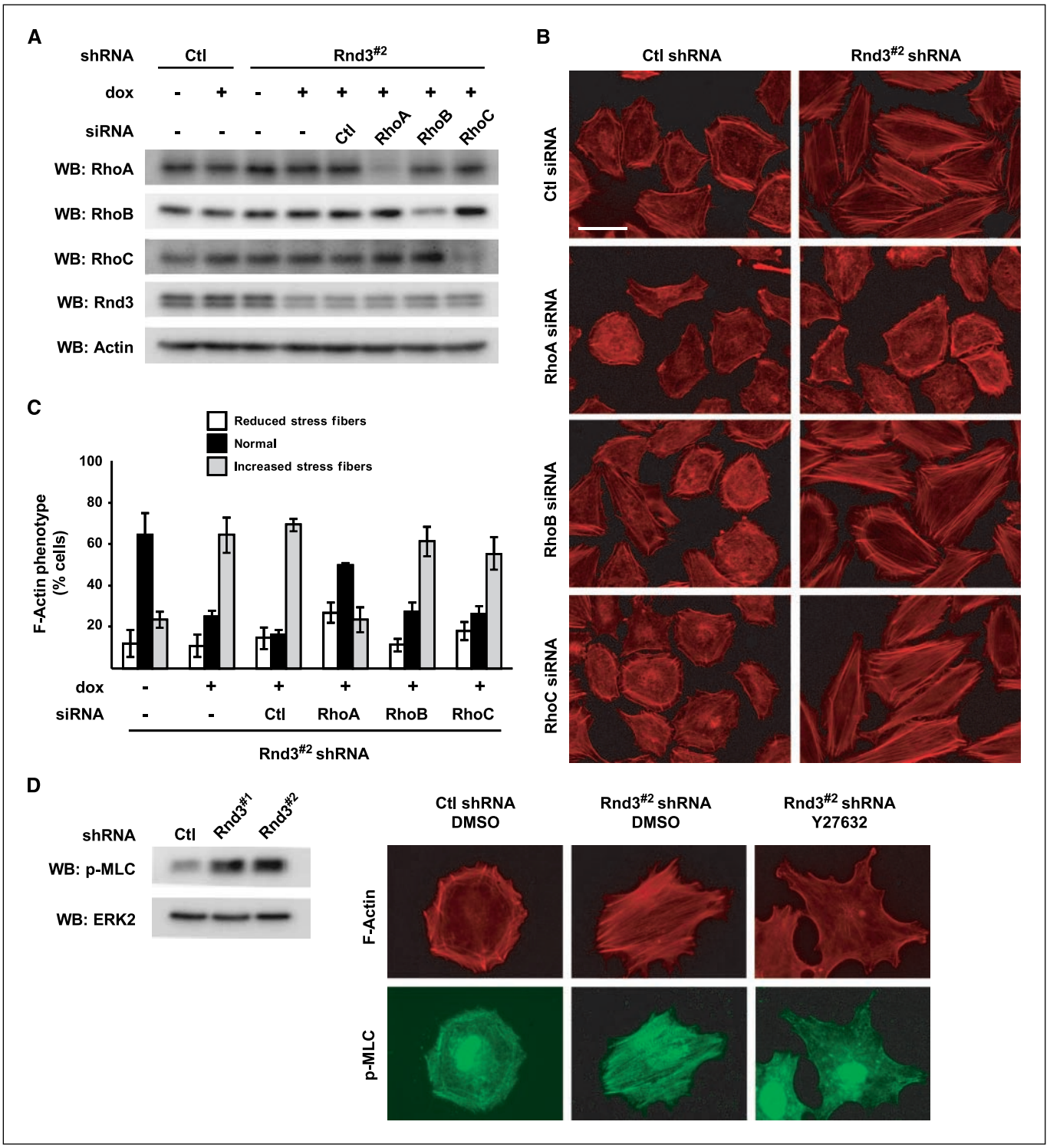

Figure 2. RhoA-ROCK signaling is required for Rnd3-dependent differences in actin cytoskeletal organization. *A* to *C,* inducible WM793TR/Rnd3$^{\#2}$ shRNA cells +/− doxycycline were transfected with siRNA duplexes targeting RhoA, RhoB, or RhoC. *A,* cell extracts subjected to Western blot analysis with antibodies specific for RhoA, RhoB, RhoC, Rnd3, or β-actin. *B,* cells fixed and processed to visualize F-actin organization. *Bar,* 50 μm. *C,* quantitation of the F-actin phenotype shown in *B*. *D,* Rnd3$^{\#2}$ and control shRNA cells incubated with doxycycline for 72 h. Cells were lysed and analyzed by Western blot for phospho-myosin light chain (*p-MLC*) and ERK2 or treated for an additional 2 h with 5 μmol/L Y27632 or equal volume of DMSO. Cells were then fixed and processed for indirect immunofluorescence using phospho-myosin light chain (*green*) and TRITC-phalloidin to visualize F-actin (*red*). *Bar,* 50 μm.

(Supplementary Fig. S4*A* and *B*). The lack of effect on the cell cycle is supported by the finding that cyclin D1 expression was not decreased in Rnd3-silenced cells (Supplementary Fig. S4*C*). Moreover, no alteration in cell growth accompanied Rnd3 knockdown (Fig. 3*C*). These data indicate that Rnd3 knockdown does not alter the proliferation or survival properties of melanoma cells.

Rnd3 expression correlates with invasive properties. The functional relationship between Rnd3 expression and the invasive

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properties of melanoma cells has not been addressed. Therefore, we next evaluated the expression of Rnd3 in normal human epidermal melanocytes as well as in noninvasive (SBcl2 and WM35) and invasive (WM115 and WM793) human melanoma cell lines. Normal human epidermal melanocytes harbor wild-type N-RAS and B-RAF; Sbcl2 express active N-RAS; and WM35, WM793, and WM115 express mutant forms of B-RAF. Notably, only the invasive cell lines showed elevated ERK1/2 phosphorylation (Fig. 4*A*). These findings are consistent with previous reports showing the presence of negative feedback loops in noninvasive melanoma cells to suppress ERK activation (6, 7). Rnd3 expression levels were also elevated in the invasive WM793 and WM115 cell lines (Fig. 4*A*).

Following invasion through the basement membrane, melanoma tumors encounter the type I collagen–rich microenvironment of the dermis. An assay used to test for invasive behavior in a dermal-like microenvironment is the nonfibrillar three-dimensional collagen spheroid outgrowth assay (37, 42). Melanoma spheroids formed from both WM35 (low Rnd3 expression) and WM793 (high Rnd3 expression) cells increased in size over 4 days (Fig. 4*B*), again suggesting that Rnd3 is not required for melanoma growth. However, WM793 and WM115 spheroids exhibited cells that progressively infiltrated into the surrounding collagen gel (Fig. 4*B* and data not shown). In contrast, WM35 spheroids did not display individual cells that invaded into the collagen gel (Fig. 4*B*). SBcl2 cells did not form compact spheroids (data not shown). Quantitation of overall invasive outgrowth after 4 days indicated that the total surface area of WM793 spheroids increased ~20-fold. By contrast, WM35 spheroids increased their area of outgrowth only ~2-fold (Fig. 4*C*). Collectively, these data show that expression of Rnd3 correlates with melanoma invasive behavior.

Rnd3 knockdown disrupts melanoma invasive outgrowth. The correlation between Rnd3 expression and melanoma invasive behavior led us to examine if Rnd3 was required for invasive activity. As shown in Fig. 5*A*, spheroids cultured in the

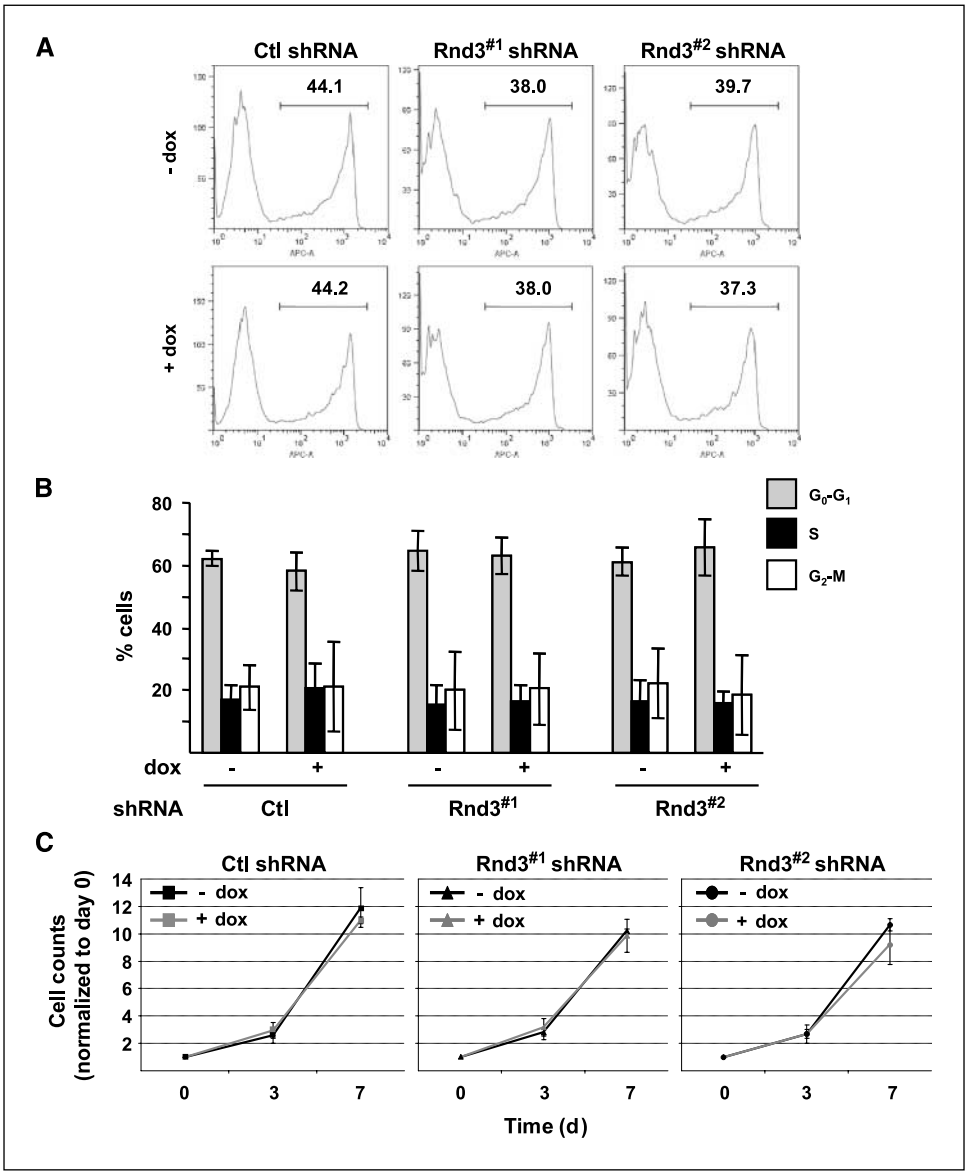

Figure 3. Rnd3 expression does not alter cell growth of mutant B-RAF–expressing invasive melanoma cells. *A,* quantitation of cell entry into S phase. Inducible knockdown cells were labeled for 7 h with EdU. DNA synthesis was analyzed by flow cytometry. Depicted are representative traces from one of three independent experiments. *B,* melanoma cells cultured in complete medium 72 h +/− 0.1 μg/mL doxycycline to induce expression of control, Rnd3$^{\#1}$, or Rnd3$^{\#2}$ shRNA. DNA was stained with propidium iodide and analyzed by flow cytometry. *Columns,* mean percentage of cells in G_0-G_1, S, and G_2-M phases of the cell cycle from three independent experiments; *bars,* SD. *C,* cell growth of inducible knockdown cells cultured in complete growth medium +/− doxycycline. Equal numbers of cells plated at day 0 +/− doxycycline were harvested and counted at days 3 and 7. *Points,* mean from one experiment done in triplicate; *bars,* SD. A total of three independent experiments were done.

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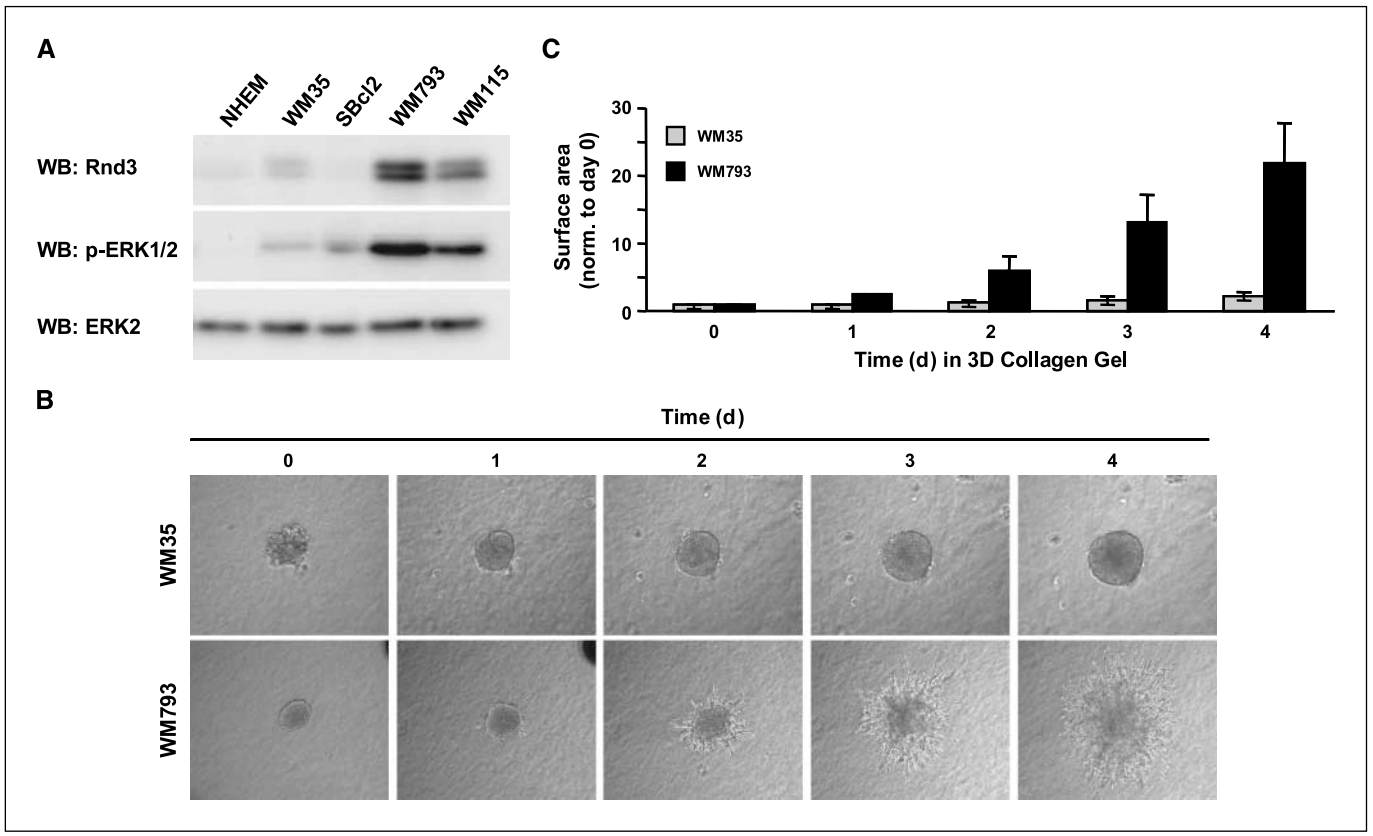

Figure 4. Increased Rnd3 expression in invasive human melanoma cells. *A,* Western blot of normal human epidermal melanocyte (*NHEM*) and noninvasive (SBcl2 and WM35) and invasive (WM793 and WM115) human melanoma cell lines for Rnd3, phospho-ERK1/2, and ERK2. *B,* micrographs depicting time course of noninvasive (WM35) and invasive (WM793) melanoma spheroids embedded in three-dimensional (*3D*) collagen gel for the indicated times. *C,* quantitation of invasive outgrowth depicted in *B*. The graph reflects total spheroid surface area measured from the same spheroids monitored over time and normalized to its size at day 0. Spheroid areas at day 0 were set to 1.

absence of doxycycline or following the induction of control shRNA displayed invasive outgrowth behavior similar to parental cells. In contrast, Rnd3 knockdown spheroids showed restricted invasive movement of cells away from the spheroid edge, whereas the area of the core spheroid remained similar to control knockdown spheroids (Fig. 5*A* and *B*). Notably, spheroid outgrowth area decreased ~10-fold in Rnd3 knockdown conditions (Fig. 5*B*). The spheroids were predominately composed of live cells with no noticeable accumulation of dead cells (Fig. 5*C*). These data show a requirement for Rnd3 in the invasive outgrowth behavior of melanoma cells.

Rnd3 regulates melanoma cell motility on three-dimensional matrices. The reduced invasive capacity accompanying Rnd3 depletion could result from defects in cell migration and/or extracellular matrix remodeling. Because Rnd3 knockdown affected actin cytoskeletal organization, which is known to affect cell motility, we next determined by video microscopy whether Rnd3 depletion altered cell migration. Initially, we evaluated Rnd3 depletion on migration of cells at the edge of multicellular spheroids overlaid onto three-dimensional collagen gels (Fig. 6*A*). Control spheroids migrated outward as a collective cell unit, increasing spheroid surface area ~3-fold after 5 hours (Fig. 6*A*). In contrast, the movement of cells out from Rnd3-depleted spheroids was attenuated (Fig. 6*A*). Overall, collective cell migration was reduced by an average of 45% in Rnd3$^{\#1}$ and 60% in Rnd3$^{\#2}$ silenced cells compared with non–doxycycline-treated cells (Fig. 6*B*). Additionally, the number of individual border cells migrating away from the expanding collective cell sheet exiting the spheroid over the 5-hour time period was consistently reduced in the absence of Rnd3. Quantitation of the number of migrating border cells revealed a 58% reduction in Rnd3$^{\#1}$ shRNA–expressing cells and a significant 67% decrease in Rnd3$^{\#2}$ shRNA–expressing cells (Fig. 6*C*). Knockdown cells displayed no differences in cell surface expression of collagen binding integrins or cell adhesion to collagen gels (Supplementary Fig. S3*A* and S3*B*). Results from F-actin organization indicated that Rnd3 knockdown–induced changes in cytoskeletal organization could be reversed on ROCK1/2 inhibition. Therefore, Rnd3-depleted cells were treated with Y27632 to determine if ROCK1/2 inhibition would prevent the reduction in cell migration on collagen gels. Pretreatment with Y27632 of Rnd3 knockdown spheroids 1 hour before plating on top collagen gel rescued collective cell migration after 5 hours (Fig. 6*D*). Collectively, these data highlight the function of endogenous Rnd3 to inhibit the Rho-ROCK signaling pathway to promote the migration of invasive melanoma cells on three-dimensional collagen matrices.

Discussion

The frequent mutational activation of B-RAF in melanoma coupled with the growing number of studies showing its significant role in melanoma initiation and progression has

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validated B-RAF as an important therapeutic target. Although clinical grade RAF inhibitors have been developed (43), their effective use to date has been diminished due to pharmacokinetic and specificity concerns (44). In addition, recent evidence suggests that increased C-RAF expression may provide for a mechanism of acquired drug resistance (45). Our studies have focused on delineating mutant B-RAF regulated targets involved in malignant traits. Complicating the analysis of these targets are the varying cellular contexts used. In this study, we analyzed the functional role of the B-RAF regulated GTPase Rnd3 in invasive melanoma cells. For the first time, our study addresses three critical issues pertaining to the role of Rnd3 in melanoma. Initially, we show that inducible knockdown of endogenous Rnd3 regulates actin cytoskeletal organization in a manner dependent on RhoA but not on RhoB or RhoC. Next, we show that Rnd3 expression is elevated in melanoma cells that display invasive outgrowth from tumor spheroids. Finally, silencing of Rnd3 profoundly reduced the invasive component of spheroid outgrowth in three-dimensional collagen gels and effectively blocked collective and border cell movements on three dimensions. Thus, the present work advances our knowledge of the signaling pathways influenced by Rnd3 and establishes its expression as a crucial regulator of invasive melanoma cytoskeletal organization and cell migration (Supplementary Fig. S5).

Oncogene-induced alterations in the actin cytoskeleton are observed in a variety of cancerous cell types (21, 46). Here, we show that depletion of endogenous Rnd3 promotes an increase in actin stress fibers, consistent with findings in osteosarcoma cells (32). Actin stress fiber formation results from activation of Rho GTPases and their subsequent effects on ROCK1/2 signaling (14, 26). Rnd3 was previously shown to inhibit Rho/ROCK signaling through its interaction with p190RhoGAP (47) and ROCK1 (30, 48). However, Rnd3 was recently shown to disassemble stress fibers independent of ROCK1 binding (49), which suggests that the Rnd3-p190RhoGAP interaction facilitates cytoskeletal disassembly. The RhoGAP domain of p190 RhoGAP can catalyze GTP hydrolysis of RhoA, RhoB, and RhoC (50). Currently, there is limited information addressing the potential isoform-specific role(s) for Rnd3 regulation of RhoA, RhoB, and RhoC. Moreover, RhoA, RhoB, and RhoC can all mediate stress fiber formation (26) and all are expressed in melanoma. The current article is the first to directly examine the contribution of

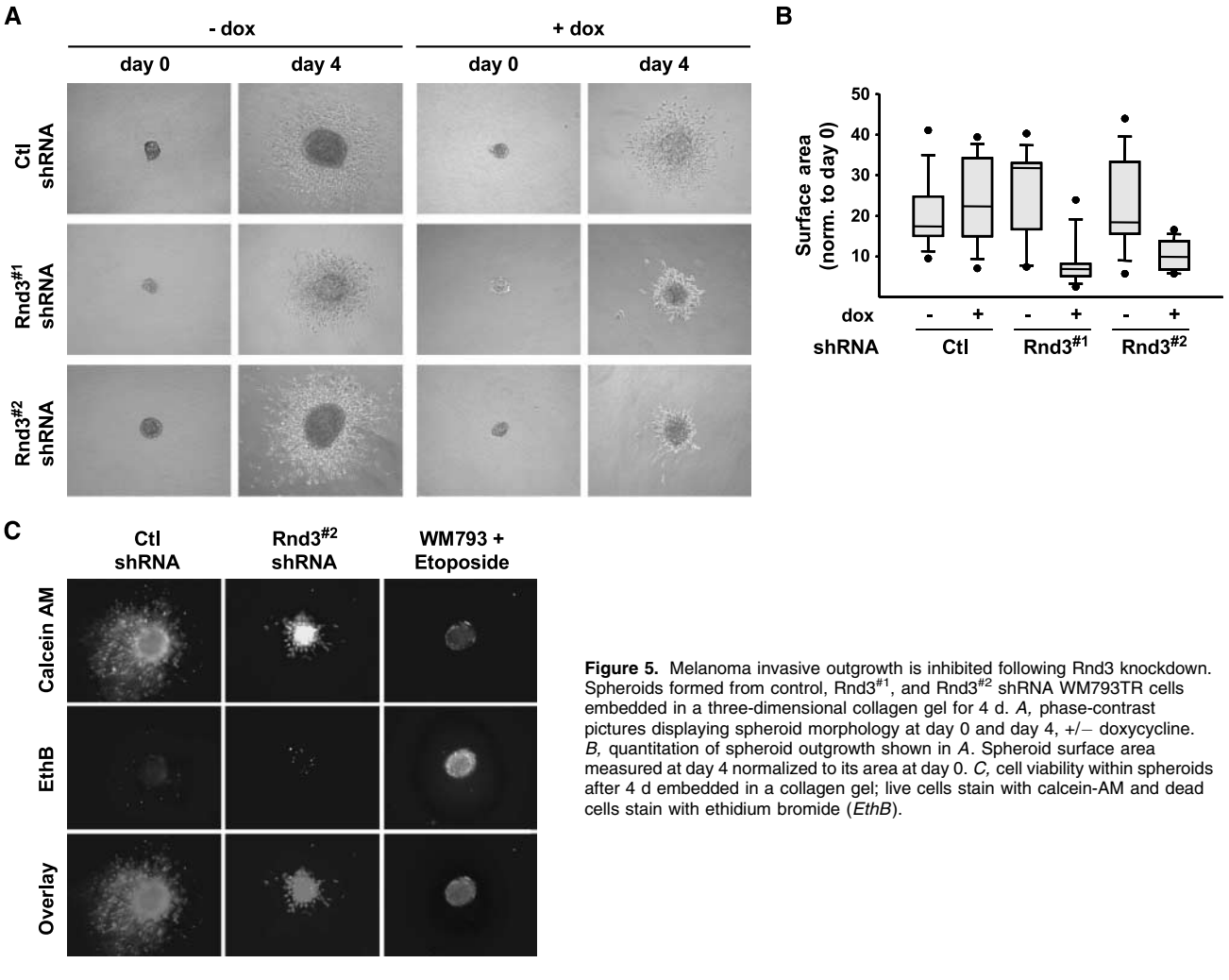

Figure 5. Melanoma invasive outgrowth is inhibited following Rnd3 knockdown. Spheroids formed from control, Rnd3$^{\#1}$, and Rnd3$^{\#2}$ shRNA WM793TR cells embedded in a three-dimensional collagen gel for 4 d. *A,* phase-contrast pictures displaying spheroid morphology at day 0 and day 4, +/− doxycycline. *B,* quantitation of spheroid outgrowth shown in *A*. Spheroid surface area measured at day 4 normalized to its area at day 0. *C,* cell viability within spheroids after 4 d embedded in a collagen gel; live cells stain with calcein-AM and dead cells stain with ethidium bromide (*EthB*).

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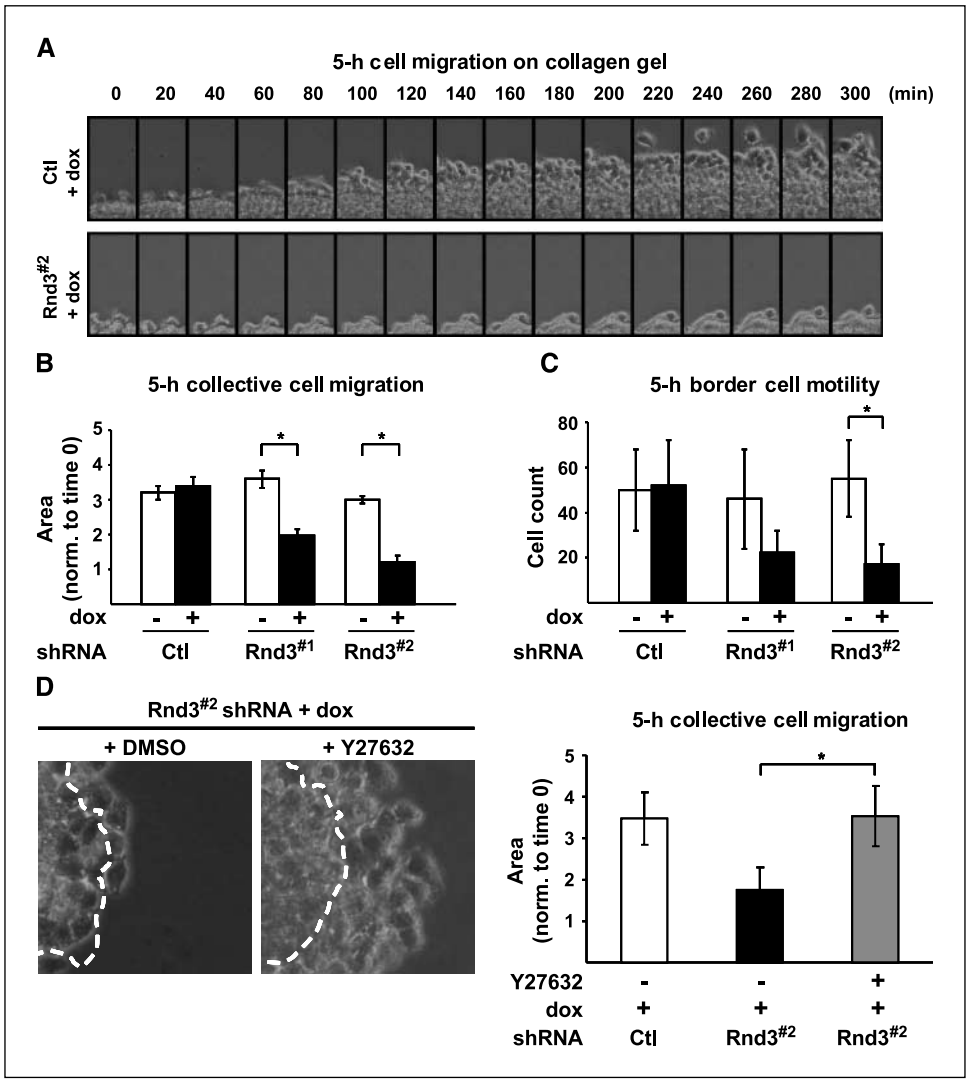

Figure 6. Rnd3 expression is required for directed melanoma cell migration on three-dimensional collagen gels. Spheroids from control, Rnd3$^{\#1}$, and Rnd3$^{\#2}$ shRNA cells cultured in complete medium +/− doxycycline on top of a collagen gel. *A,* micrographs of spheroids acquired from time lapse microscopy over a 5-h imaging period. *B* and *C,* quantitation of spheroid migration depicted in *A. B,* collective cell movement, measured as the increase in spheroid surface area over time (*, $P < 0.05$). *C,* the number of border cells, determined by counting individual cells separated from the cell sheet (*, $P < 0.05$). *D,* collective cell movement of control and Rnd3$^{\#2}$ shRNA cells treated +/− 5 μmol/L Y27632 at 1 h before plating on collagen gels. Images show an overlay of the spheroid surface area at time 0 (outlined by white line) superimposed onto its area 5 h later. Graph depicting quantitation of the collective cell movement in spheroids treated +/− Y27632 (*, $P < 0.05$).

RhoA, RhoB, and RhoC isoforms to Rnd3-mediated cytoskeletal effects at the molecular level. Our results show that Rnd3 preferentially restricts RhoA signaling to regulate actin organization. This is consistent with a previous report in breast carcinoma showing that the roles of RhoA, RhoB, and RhoC are not redundant (17). Whereas our studies in vertical growth phase melanoma cells implicate inhibition of RhoA signaling in the control of actin stress fibers and cell migration, we cannot rule out a requirement for additional Rho isoforms during the later stages of melanoma progression.

In nonmelanoma cell types, Rnd3 expression has been linked to either increased or decreased cell proliferation (29, 40) and prosurvival effects (32). In invasive melanoma that expresses mutant B-RAF, we did not observe changes in the cell cycle profile or growth following Rnd3 knockdown. Furthermore, the levels of ERK1/2 and AKT phosphorylation, which regulate melanoma proliferation and survival, as well as cyclin D1 expression, were similar between the control and Rnd3-depleted melanoma cell lines. Thus, it seems that the relationship between Rnd3 expression and cell proliferation varies between different types of cells. One explanation for these discrepancies may be that mutant B-RAF signaling in melanoma overrides any change in cell growth imposed by Rnd3.

The seemingly contradictory studies on Rnd3 expression in cancer (19, 22, 23) as well as our previous results showing induction of Rnd3 by mutant B-RAF (21) prompted us to examine the expression of Rnd3 in cells characteristic of noninvasive and invasive stages of melanoma. Data presented here show that increased Rnd3 expression correlated with the transition from noninvasive to invasive melanoma. Interestingly SBcl2 and WM35 cells, which harbor mutant N-RAS and mutant B-RAF, respectively, displayed low Rnd3 expression. This is likely due to the reduced phosphorylation of active ERK1/2 in SBcl2 and WM35. Differences in ERK1/2 activation in melanoma cells that harbor mutant B-RAF may be due to amplification of mutant B-RAF alleles in invasive cells or the presence of a negative feedback loop in noninvasive cells (6, 7). The mechanism of ERK1/2 regulation of Rnd3 in melanoma is largely unknown. Rnd3 has recently been reported to be a p53 transcriptional target gene (32); however, knockdown of wild-type p53 in WM793 cells did not disrupt Rnd3 expression (Supplementary Fig. S6). Now that a role for Rnd3 in invasive melanoma has been established, additional studies are warranted

to identify the mechanism(s) responsible for ERK1/2 control of Rnd3 expression. In sum, it seems that Rnd3 expression in melanoma is associated with elevated B-RAF/MEK/ERK signaling as well as a proinvasive phenotype.

Vertical growth phase melanoma is distinguished from radial growth phase melanoma by its invasive movement into the dermis (1). In a three-dimensional model system, we show that knockdown of endogenous Rnd3 dramatically attenuates the ability of spheroid cells to invade out into the surrounding collagen gel. Notably, the spheroid core increased in size and was composed of live cells, suggesting that overall growth was maintained. Analysis of spheroids placed on top of collagen gels revealed that migration of collective cell layers, as well as individual border cells, was reduced in Rnd3-depleted spheroids. Although a precise mechanism for reduced migration in three-dimensional matrices is currently under investigation, the results from two-dimensional assays implicate deregulated RhoA signaling. This would be consistent with current reports implicating Rho/ROCK signaling in the regulation of tissue compliance and tumor cell migration (10, 13, 36). Future studies will examine whether Rnd3 regulation of RhoA facilitates melanoma migration in three-dimensional matrices through its control of focal adhesion dynamics, extracellular matrix protease activity, and/or cellular tension.

In conclusion, the current data advance our knowledge of Rnd3 and Rho GTPases in melanoma. Increased expression of Rnd3 attenuates RhoA-ROCK signaling in invasive melanoma to regulate three-dimensional migration. Our results potentially serve as the framework for new therapeutic strategies that target oncogene-regulated effector pathways to reduce invasive behavior and restrict malignancy in human cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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R. Matthew Klein and Andrew E. Aplin

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